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TITLE: The Role of TSC2 Phosphorylation in the Regulation of TSC2 Localization and mTOR Signaling

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14. ABSTRACT
Tuberous sclerosis is a genetic disease that affects an estimated 1 in 6,000 births, and it occurs when mutations in components of the tuberous sclerosis complex (TSC) render it functionally inactive. Research over the last decade has established that the TSC controls the activity of a signaling molecule called mTOR, and aberrant regulation of the TSC/mTOR pathway has become widely implicated in the pathogenesis of tuberous sclerosis. However, the mechanism(s) through which the TSC regulates mTOR signaling have not been resolved. Our overall hypothesis predicts that agonists, such as mechanical signals, regulate mTOR signaling by inducing a phosphorylation-dependent loss in the association of TSC2 with late endosomal / lysosomal system. During the first year of this project we have successfully identified 6 phosphorylation sites on TSC2 that are regulated by mechanical stimuli. We then generated phosphodefective mutants of TSC2 and our preliminary results with these mutants indicate that these phosphorylation sites play a central role in the pathway through which mechanical stimuli regulate mTOR signaling.

15. SUBJECT TERMS
Colocalization, Electroporation, GAP, Insulin, LAMP2, Late Endosome, Lysosome, Mass Spectrometry, Mechanical, mTOR, $p70^{S6k}$, Phosphorylation, Rheb, Transfection, TSC2, Tuberous Sclerosis.

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1. INTRODUCTION:

Tuberous sclerosis is a genetic disease that affects an estimated 1 in 6,000 births, and it occurs when mutations in components of the tuberous sclerosis complex (TSC) render it functionally inactive. Research over the last decade has established that the TSC controls the activity of a signaling molecule called mTOR, and aberrant regulation of the TSC/mTOR pathway has become widely implicated in the pathogenesis of tuberous sclerosis. However, the mechanism(s) through which the TSC regulates mTOR signaling have not been resolved. Therefore, the primary objective of this project is to develop a better understanding of these mechanisms.

2. KEYWORDS:

Colocalization, Electroporation, GAP, Insulin, LAMP2, Late Endosome, Lysosome, Mass Spectrometry, Mechanical, mTOR, $p70^{S6k}$, Phosphorylation, Rheb, Transfection, TSC2, Tuberous Sclerosis.

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
- **What was accomplished under these goals?**
- **What do you plan to do during the next reporting period to accomplish the goals?**

For the sake of simplicity we have responded to these three questions by inserting the original statement of work (in Times New Roman font) and then indicating the percentage of progress that has been made on each task / subtask. At the end of each major task we have included a section that briefly summarizes our accomplishments, setbacks, and our plans for the second year of the project period.

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Statement of Work

Task 1: Goal

Identify the Phosphorylation Sites on TSC2 that are Regulated by Insulin and Mechanical Stimulation (Aim 1).

Subtask 1a. Generate electroporation grade plasmid DNA encoding wild type FLAG-tagged TSC2 ($TSC2^{WT}$) and then electroporate / transfect the TA muscles of mice$^{\surd}$ (n = 4 / group for control, insulin stimulation and mechanical stimulation conditions, total = 12 mice). Note: this grant application has a 5 reference limit, so the $^{\surd}$ symbol is being used to identify methods that have already been established by our group.

≈70% complete

Subtask 1b. Subject the electroporated mice to insulin stimulation, mechanical stimulation, or the control condition at 7 days post transfection$^{\surd}$. Immunopurify the FLAG-tagged TSC2, separate by SDS-PAGE, and then subject the TSC2 band to in-gel digest followed by LC/MS/MS analysis$^{\surd}$.

≈70% complete

Subtask 1c. Analyze MS data and perform label-free quantification on the identified phosphorylation sites$^{\surd}$. Use literature, database and bioinformatics based searches to gain insight into the potential role of each phosphorylation site$^{\surd}$.

≈70% complete

Task 1: Accomplishments, Setbacks and Future Plans

To date we have completed our analyses that were aimed at identifying the phosphorylation sites on TSC2 that are regulated by mechanical stimulation. As shown in Figure 1, our analyses led to the identification of 16 different phosphorylation sites on TSC2, and we discovered that the phosphorylation of 6 of these sites was significantly elevated following a bout of mechanical stimulation. Two of the regulated sites that were identified

(Ser1254 and S1364) were found to lie within RxRxxS*/T* consensus motifs, and the remaining four sites fell within proline-directed motifs (S*/T*P).

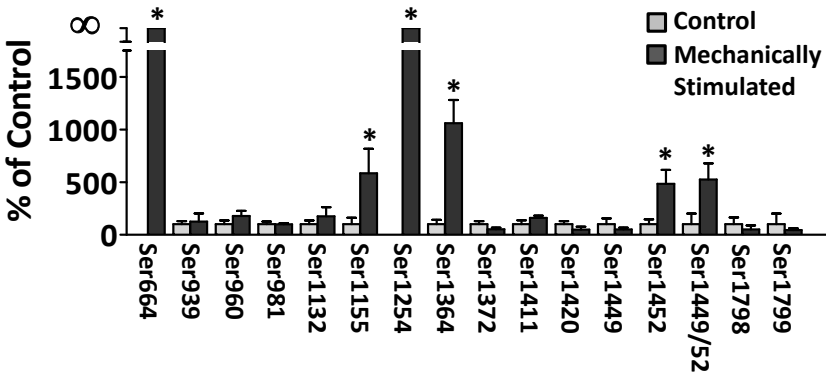

Figure 1. Quantification of the different phosphorylation sites identified on TSC2 in control and mechanically stimulated muscles. All values are expressed as a percentage of the values obtained in the control samples. In some instances, the phosphorylation site was not identified in control samples, and thus, the value in mechanically stimulated samples is represented as infinity (∞). All values are presented as the mean + SEM, n = 6 / group. * Significantly different from control, $P < 0.05$.

To date, we have not completed our analysis of insulin regulated phosphorylation sites. Our delay in this progress stems from the fact that the generation of the mechanical stimulation data set alone exhausted far more resources than we had anticipated. For instance, we originally expected that all of the control, insulin and mechanical stimulation data could be generated from 4 independent experiments containing one biological replicate per group. However, by the completion of our third experiment it became apparent that this was an overly optimistic expectation. Therefore, we refined our efforts and focused specifically on the effects of mechanical stimulation. In total, the generation of the mechanical stimulation data set ended up requiring 9 independent experiments. Various factors contributed to this need including: a) insufficient protein isolation during the immunoprecipitation procedure; b) insufficient protein isolation during the gel excision procedure; c) poor recovery of peptides following trypsin digestion; and d) an n = 6 per group was needed before all apparent changes were determined to be significantly different (we originally anticipated that this would require n = 4 / group). Thus, although we were successful in identifying the phosphorylation sites that are regulated by mechanical stimulation, we have yet to complete our analyses of the sites that are regulated by insulin stimulation. Given that many of the insulin regulated phosphorylation sites have already been reported, we shifted our efforts towards the completion of tasks #2-6 and plan to return to task #1 during the second year of the project.

Task 2: Goal

Determine if the Identified Phosphorylation Sites on TSC2 Regulate its GAP Activity Towards Rheb *In-Vivo* (Aim 2a).

*Subtask 2a***.** Use site directed mutagenesis to generate the FLAG-tagged $TSC2^{INS}$, $TSC2^{MCH}$, $TSC2^{INS\&MCH}$ and $TSC2^{5A}$ mutations on the original FLAG-tagged $TSC2^{WT}$ construct√. Subclone the different TSC2 constructs into the pIRESneo3 vector for the generation of stable cell lines√.

≈ 40% complete

Subtask 2b. Stably reconstitute $TSC2^{-/-}$ MEFs with the $TSC2^{WT}$, $TSC2^{INS}$, $TSC2^{MCH}$, $TSC2^{INS\&MCH}$, and $TSC2^{5A}$ constructs described in 2a, and screen for pools of cells that express the reconstituted TSC2 at a level that is comparable to that found in wild type MEFs√.

≈ 10% complete

Subtask 2c. Subject the different stably reconstituted $TSC2^{-/-}$ MEFs lines to insulin stimulation, mechanical stimulation or the control condition√, and measure the *in-vivo* Rheb GAP activity.

≈ 10% complete

Task 2: Accomplishments, Setbacks and Future Plans

In order to complete this, and all of the remaining tasks, we needed to create phosphodefective mutants of TSC2. To date we have created a construct that contains mutations on all of the 6 phosphorylation sites that were found to be responsive to mechanical stimulation ($TSC2^{MCH}$). We have also obtained a TSC2 construct ($TSC2^{5A}$) that contains mutations on five of the phosphorylation sites that are known to be regulated by insulin. Our original plan was to create a series of stable cell lines that express these mutant proteins (subtask 2b), and then use these stable cell lines to determine the role that the phosphorylation sites play in controlling the ability of TSC2 to function as a GAP for Rheb *in-vivo*. Importantly though, the measurement of TSC2's GAP activity *in-vivo* is dependent on our ability to successfully immunoprecipitate Rheb. Therefore, we first focused on the optimization of a Rheb immunoprecipitation procedure. So far, in these experiments we have employed three different antibodies, as well as an antibody that purportedly can immunopurify active (GTP-bound) Rheb. Unfortunately, none of the antibodies that we have tested were able to immunopurify Rheb at a level that was significantly greater than that found in negative control samples. Despite this setback, we plan to continue trying new antibodies and hope to get this assay working during the second year of the project. If we are able to get the assay working then we will complete the remainder of task #2 as originally described.

Task 3: Goal

Determine if the Identified Phosphorylation Sites on TSC2 Regulate its GAP Activity Towards Rheb *In-Vitro* (Aim 2b).

Subtask 3a. Purify GST-Rheb and load with [γ-^{32}P]GTP for the performance of the *in-vitro* Rheb GAP activity assay.

≈ 30% complete

Subtask 3b. Subject the different stably reconstituted $TSC2^{-/-}$ MEFs lines to insulin stimulation, mechanical stimulation or the control condition$^{\surd}$. Collect the cells, immunopurify the TSC complex$^{\surd}$, and perform the *in-vitro* Rheb GAP activity assay.

- No progress, this subtask will be initiated after the completion of subtask 3a.

Task 3: Accomplishments, Setbacks and Future Plans

To date, we have successfully obtained all of the reagents that are needed to complete this task, and we are currently in the process of moving forward with subtask 3a. Once completed, we will move on to subtask 3b.

Task 4: Goal

Determine if the Identified Phosphorylation Sites on TSC2 Regulate the Insulin- and Mechanically-Induced Dissociation of TSC2 from the LEL (Aim 3a).

Subtask 4a. Generate electroporation grade plasmid DNA encoding the different FLAG-TSC2 constructs described in 2a, and then electroporate / transfect the TA muscles of mice$^{\surd}$ [n = 5 plasmid conditions ($TSC2^{WT}$, $TSC2^{INS}$, $TSC2^{MCH}$, $TSC2^{INS\&MCH}$, $TSC2^{5A}$), and n = 4 / group within the insulin stimulation, mechanical stimulation, and control conditions, thus n = 12 / plasmid condition, and the total n = 60 mice].

≈ 40% complete

Subtask 4b. Subject the electroporated mice to insulin stimulation, mechanical stimulation, or the control condition at 7 days post transfection and save the TA muscles for immunohistochemical analysis$^{\surd}$.

≈ 40% complete

Subtask 4c. Generate semi-ultrathin cross-sections from the TA muscles in 4b$^{\surd}$. Perform immunohistochemistry for TSC2 and LAMP2, and then perform colocalization analysis as described in Fig. 1 of the project narrative$^{\surd}$.

≈ 10% complete

Task 4: Accomplishments, Setbacks and Future Plans

Our original statement of work indicated that we would initiate task 4 during the second year of the project period. However, we have already started to make progress on this task. For instance we have generated the electroporation grade DNA for the $TSC2^{WT}$, $TSC2^{MCH}$ and $TSC2^{5A}$ constructs and performed preliminary experiments on muscles that were transfected with these constructs. We have also generated control and mechanically stimulated samples that have been transfected with the $TSC2^{WT}$ construct (n = 2 / group), as well as control and mechanically stimulated samples that have been transfected with the $TSC2^{MCH}$ construct (n = 2 / group). These samples were then successfully subjected to immunohistochemical analysis for TSC2 and LAMP2. Based on the images obtained we were able to reaffirm that the originally described colocalization methodology could be used to obtain the colocalization data. Moreover, our school recently obtained a new Leica SP8 confocal microscope and we were successfully able to adapt and optimize our immunohistochemical procedures for this microscope. Moreover, we determined that the quality of the images obtained with the Leica microscope were superior to those obtained with our original microscope. Therefore, we are now in the process of generating the remaining samples for our data sets and will initiate our colocalization analyses on the Leica microscope once these samples have been generated.

Task 5: Goal

Determine if the Identified Phosphorylation Sites on TSC2 Regulate the Insulin- and Mechanically-Induced Activation of mTOR Signaling (Aim 3b).

Subtask 5a. Generate electroporation grade plasmid DNA encoding myc-tagged $p70^{S6k}$ √.

≈ 100% complete

Subtask 5b. Electroporate / co-transfect the TA muscles of mice with myc-$p70^{S6k}$ and the different FLAG-TSC2 constructs described in 2a√ [n = 5 plasmid conditions ($TSC2^{WT}$, $TSC2^{INS}$, $TSC2^{MCH}$, $TSC2^{INS\&MCH}$, $TSC2^{5A}$), and n = 4 / group within the insulin stimulation, mechanical stimulation, and control conditions, thus n = 12 / plasmid condition, and the total n = 60 mice].

≈ 40% complete

Subtask 5c. Subject the electroporated mice to insulin stimulation, mechanical stimulation, or the control condition at 7 days post transfection and save the TA muscles for immunoprecipitations and Western blot analysis√.

≈ 40% complete

Task 5: Accomplishments, Setbacks and Future Plans

Our original statement of work indicated that we would initiate task 5 during the second year of the project period. Nonetheless, we have already made significant progress on this task. Specifically, we have generated control and mechanically stimulated samples from muscles that were co-transfected with myc-$p70^{S6k}$ and the $TSC2^{WT}$ construct (n = 6 / group), as well as samples from muscles that were co-transfected with myc-$p70^{S6k}$ and the $TSC2^{MCH}$ construct (n = 7 / group). We then immunopurified the myc-$p70^{S6k}$ and performed Western blot analyses to determine the phospho (T389) to total protein ratio on myc-$p70^{S6k}$ as a marker of mTOR signaling. As shown in figure 2, the results from these analyses suggest that the magnitude of the mechanical activation of mTOR signaling is reduced in muscles that express $TSC2^{MCH}$ when compared with muscles that express $TSC2^{WT}$. These results are consistent with our overall hypothesis and suggest that the mechanically-regulated phosphorylation sites on TSC2 play a central role in the pathway through which mechanical stimuli activate mTOR signaling. However, the p-value for this comparison is currently at 0.10, hence, we will need to generate additional samples before any final conclusions can be reached. Once completed, we will then move forward with the remainder of the experiments described in this task.

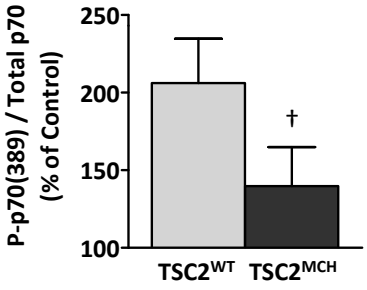

Figure 2. Muscles were co-transfected with myc-p70^{S6k} and either the TSC^{WT} or TSC^{MCH} constructs. The muscles were allowed to recover for 7 days and then were subjected to a bout of mechanical stimulation or the control condition. The myc-p70^{S6k} from these samples was immunoprecipitated and then subjected to Western blot analysis to determine the phosphorylated to total protein ratio on myc-p70^{S6k} [P-p70(389) / Total p70]. This ratio was then expressed as a percentage of the mean value obtained in the construct matched control muscles. The data in the graph represents the values that were observed in the muscles subjected to mechanical stimulation. All values are presented as the mean + SEM, n = 6-7 / group. † P = 0.10.

Task 6: Goal

Draft manuscript on "The Role of TSC2 phosphorylation in the regulation of TSC2 localization and mTOR signaling".

≈ 10% complete

Task 6: Accomplishments

We have developed a preliminary plan for the layouts of the figures that will likely be included in the manuscript.

- **What opportunities for training and professional development has the project provided?**

The project provides support (75% effort) for a pre-doctoral graduate student (Brittany L. Jacobs, 75% effort). Thus, by working with the PI on this project, Ms. Jacobs has been able to become more proficient in her abilities to function as an independent scientist.

- **How were the results disseminated to communities of interest?**

The PI presented some of the preliminary results from the MS analyses (Fig. 1) during a talk that he delivered at the Integrative Physiology of Exercise conference (September 2014, Miami, FL), and during a local talk that was given to the Cell and Regenerative Biology program at the University of Wisconsin - Madison (November 2014, Madison, WI). The PI also presented some of the preliminary data from the mechanical stimulation experiments (Fig. 2) during a talk that was delivered at the Exercise Science and Health meeting (March 2015, Cold Springs Harbor, NY). The graduate student also presented these data in posters and abstracts at the University of Wisconsin MCP 12th Annual Signal Transduction Symposium (March 2015, Madison, WI), and the University of Wisconsin School of Veterinary Medicine Phi Zeta Research Day (April 2015, Madison, WI).

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

Our preliminary data (Fig. 2) suggests that changes in the phosphorylation of TSC2 contribute to the signaling pathway through which mechanical stimuli regulate mTOR. If this holds true, then our identification of the phosphorylation sites that are regulated by mechanical stimuli (Fig. 1) would be expected to have a fairly broad impact on the fields of tuberous sclerosis and mTOR signaling. We say this because the functional significance of several of the identified phosphorylation sites (S1155, S1364, S1449 and S1452) has never been explored. Importantly, with our experimental approach we will ultimately be able to identify which specific phosphorylation sites contribute to the regulatory effect. This information could be fundamentally important for the field and it will also provide the foundation for future studies that are aimed at understanding how changes in TSC2 phosphorylation control its ability to regulate mTOR signaling. According to our hypothesis, changes in the phosphorylation of TSC2 will alter its association with the late endosomal / lysosomal (LEL) system, and testing this hypothesis will be an exciting area of our focus in the second year of the project.

- **What was the impact on other disciplines?**

In addition to the potential impact on the fields of tuberous sclerosis and mTOR signaling, we expect that our findings will also advance our understanding of how mechanical signals (e.g., resistance exercise) regulate skeletal muscle mass. The basis for this argument stems from previous studies which have shown that: 1) mechanical stimuli cause a robust activation of mTOR signaling; 2) signaling through mTOR is necessary for a mechanically-induced increase in muscle mass: and 3) the activation of mTOR signaling is sufficient to induce an increase in muscle mass. Hence, it is apparent that the activation of mTOR signaling plays a central role in the pathway through which mechanical stimuli regulate muscle mass. Therefore, by defining how mechanical stimuli activate mTOR, we will advance the field's understanding of how mechanical stimuli regulate skeletal muscle mass.

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change.**
- **Actual or anticipated problems or delays and actions or plans to resolve them.**

There are no significant changes to report; however, some minor changes occurred during the first year of the project. Details with regards to the reason for these changes are described in question #3. To very briefly summarize, our original statement of work indicated that we would have completed tasks 1-3 by the end of the first year. For the reasons outlined in question 3, we have not fully completed these tasks, but we anticipate that these tasks will be completed before the end of the project period. In addition, our original statement of work indicated that the work on tasks 4-6 would not initiated until the second year of the project period. However, as described in question 3 we have already made significant, and promising, progress on these tasks. Thus, while the completion of the individual tasks has deviated from the originally described timeline, we believe that our overall progress is moving along exactly as we would have hoped.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.**

Nothing to Report

- **Significant changes in use or care of human subjects**

Not Applicable

- **Significant changes in use or care of vertebrate animals.**

Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report

Books or other non-periodical, one-time publications.

Abstracts:

1. Jacobs BL, Kim KJ, Hornberger TA. A Role for TSC2 in the Mechanical Regulation of mTOR Signaling. *University of Wisconsin School of Veterinary Medicine Phi Zeta Research Day 2015.* Madison, WI. Abstract and Poster.
2. Jacobs BL, Kim KJ, Hornberger TA. TSC2 is Necessary for the Maximal Mechanical Activation of mTOR Signaling. *University of Wisconsin MCP 12th Annual Signal Transduction Symposium 2015.* Madison, WI. Abstract and Poster.

Other publications, conference papers, and presentations.

Presentations:

1. The Integrative Biology of Exercise Conference VII. Speaker and Chair of the Session. Mechanotransduction and the Regulation of Skeletal Muscle Mass. Miami FL, September 18th, 2014.
2. Department of Cell and Regenerative Biology University of Wisconsin - Madison. The Mechanical Activation of mTOR Signaling: An Emerging Role for Lysosomal Targeting. Madison WI, November 14th, 2014.
3. Exercise Science & Health Meeting. The Regulation of Skeletal Muscle Mass by Mechanically-Induced Signaling Events. Cold Spring Harbor NY, March 10th, 2015.

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. ARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: Troy A. Hornberger
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): ORCID ID 0000-0002-2349-1899
Nearest person month worked: 1
Contribution to Project: Generated the design of the experimental procedures and monitored the progress of the research. Mentored the graduate student that conducted the experimental procedures.
Funding Support: Not Applicable

Name: Brittany L. Jacobs
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): ORCID ID 0000-0002-1078-2320
Nearest person month worked: 9
Contribution to Project: Conducted all of the primary experimental procedures and analyzed the results.
Funding Support: Not Applicable

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Yes, the NIH Research Project Grant 1R01AR057347-06 has been funded for the period of 03/01/2015 - 2/28/2020 and should now be listed as active support.

- **What other organizations were involved as partners?**

  Organization Name: The University of Wisconsin - Madison
  Location of Organization: Madison, WI
  Partner's contribution to the project: Facilities

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable

9. APPENDICES:

Not Applicable